REMARKS

Claims 1-35 are pending in the present application. Claims 15, 16, 31 and 32 have been withdrawn from consideration. Claims 1-14, 17-30 and 33-35 were considered on the merits. Claims 1 and 17 are currently amended to define Applicants' invention with greater particularity. No new matter has been added and the amended language is fully supported by the application and claims as originally filed. Applicants thank the Examiner for withdrawing the 35 U.S.C. §112 rejections from the previous Office Action. Currently, all of the claims under consideration have been rejected as obvious under 35 U.S.C. §103. Applicants respectfully traverse these rejections.

I. Claim Rejections – 35 U.S.C. §103

A. Kai *et al.* in view of Masuda *et al.*

In the Office Action, claims 1-8, 10, 14, 17-24, 26, 29-30 and 33 continue to be "rejected under 35 U.S.C. 103(a) as being unpatentable over Kai *et al.* (JP 2001 089390 A) in view of Masuda *et al.* (US 6197061 B1)." Applicants traverse this rejection. As stated in §2143 of the MPEP,

> [t]o establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

Applicants respectfully submit that a *prima facie* case of obviousness has not been established.

First, all of the elements recited in the currently pending claims have not been taught or suggested by the references cited by the Examiner. Applicants respectfully submit that contrary to the Examiner's assertion, the rapid degradation of the cartilage tissue is not an inherent property of the tissue of Masuda *et al.* As the MPEP states under section 2112, "the fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to

establish the inherency of that result or characteristic" (emphasis in original). The MPEP further states that:

> In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art.
>
> To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.

MPEP §2112 under the heading "Examiner Must Provide Rationale or Evidence Tending To Show Inherency" (emphasis in original).

Amended claims 1 and 17 distinguish over Masuda *et al.* at least by requiring the use of an engineered cartilage matrix that is cultured to be rapidly degraded, losing roughly half of its proteoglycan content within 24 hours after treatment with IL-1. Although under certain culture conditions the tissue of Masuda *et al.* may be capable of such rapid degradation, under different culture conditions the engineered tissue of Masuda *et al.* does not share this property. In fact, Masuda *et al.* expressly teaches that "mechanical properties of the cartilage matrix can be controlled by increasing or decreasing the amount of time that the cartilage tissue is cultured on the membrane. Longer culture time will result in increased crosslink densities." Col. 8, lines 41-45. Thus, the engineered cartilage of Masuda *et al.* changes with time in culture. Greater time in culture results in increased crosslink densities, making the tissue of Masuda *et al.* behave more like native cartilage tissue. Col. 7, line 15. In this form, the tissue of Masuda *et al.* cannot be used in the present methods.

In particular, when cultured to be similar to native tissue, the tissue of Masuda *et al.* lacks the ability to undergo rapid degradation. The skilled artisan will readily appreciate this fact as shown by the enclosed Augustine *et al.* reference and the previously presented Aydelotte *et al.* article. These references teach that in order to produce 50% proteoglycan release as a result of adding IL-1 to native cartilage in explant culture or in isolated chondrocytes, scientists

typically culture the explants for 4 days. Augustine *et al.*, Inflamm Res, 46: 60-64 (1997), Aydelotte *et al.*, Articular Cartilage and Osteoarthritis 237, FIG. 2 (1992). Likewise, native-like forms of the tissue of Masuda will take longer to produce 50% proteoglycan release than 24 hours. (If the Examiner would like experimental support for this statement, an affidavit from Dr. Masuda can be provided). Thus, it is evident that how the tissue of Masuda *et al.* is cultured directly influences the time frame for proteoglycan release. Rapid degradation is simply not a property that necessarily flows from the tissue of Masuda *et al.* The tissue is not like aspirin with a fixed composition and inherent properties. One must choose to culture the tissue in a specific way to obtain the property of rapid degradation for use in the claimed methods. Neither Masuda *et al.* nor Kai *et al* teach any such selection and use of the engineered cartilage.

Indeed, Applicants respectfully submit that the Examiner is confusing inherency in the cartilage tissue with inherency in a method of determining the effect of a test agent. The purpose of the rule set forth in MPEP §2145 regarding inherency is to prevent removal "from the public that which is in the public domain by virtue of its inclusion in, or obvious from, the prior art." *In re* Wiseman; 596 F.2d 1019 (CCPA 1979). However, Applicants are not attempting to patent a known method, thereby removing it from the public domain. Nor are Applicants attempting to patent an old compound for which they discovered a new property. Rather, Applicants have recognized a previously unappreciated trait found in select samples of the engineered cartilage tissue disclosed by Masuda *et al.* and taken advantage of this trait to develop the new and useful methods of the present invention. The skilled artisan can readily appreciate the advantages of the present methods, such as decreased culture costs and increased number of agents tested which results from the speed of the methods. Yet this speed is not necessarily present and does not necessarily flow from the tissue of Masuda *et al.* Independent claims 1 and 17 are method claims requiring the use of an engineered cartilage matrix that is cultured to be rapidly degraded. Masuda *et al.* does not disclose such methods and does not indicate when or how such a rapidly degraded tissue is to be used. Accordingly, use in the claimed methods of an engineered cartilage cultured to be rapidly degraded cannot be inherent to the disclosure of Masuda *et al.*

Additionally, it is respectfully submitted that there is no suggestion either in Kai *et al.* or in Masuda *et al.*, or in the knowledge generally available to one of ordinary skill in the art, to modify the culture system of Masuda *et al.* with the cartilage cells of Kai *et al.* to determine the effects of test agents on the specialized cartilage tissue of the present invention. Applicants respectfully disagree with the Examiner's assertion that "common culture practices in the art" provides motivation to combine Kai *et al.* with Masuda *et al.* Even if two teachings can be combined, that is not sufficient motivation to combine them under an obviousness analysis. See MPEP § 2143.01 under the heading "Fact That References Can Be Combined Or Modified Is Not Sufficient To Establish Prima Facie Obviousness." Moreover, as stated in §2144.03 of the MPEP, "it is never appropriate to rely solely on 'common knowledge' in the art without evidentiary support in the record, as the principal evidence upon which a rejection was based." In this case, the Office Action simply fails to provide evidence that one skilled in the art would combine the Kai *et al.* and Masuda *et al.* references. Applicants respectfully request that by either official notice or a piece of prior art, the Examiner support the statement that one of skill in the art would combine the Kai *et al.* and Masuda *et al.* references to obtain the present invention. Absent such evidentiary support to combine the references, Applicants respectfully submit that a *prima facie* case of obviousness has not been met, and the rejection must be withdrawn.

Based on the foregoing arguments, Applicants respectfully submit that because the limitation of rapid degradation in claim 1 and claim 17 is not inherent, the combination of Kai *et al.* and Masuda *et al.* does not teach every element of the claimed invention. Nor do the cited references supply any motivation for their combination. Applicants respectfully submit that a prima facie case of obviousness has not been established for claims 1 and 17. As claims 2-8, 10, 14, 18-24, 26, 29-30 and 33 all depend either directly or indirectly from claim 1 or claim 17, the cited art fails to establish a prima facie case of obviousness for these claims as well. Therefore, Applicants respectfully request that the rejection under 35 U.S.C. § 103(a) based on the combination of Masuda *et al.* and Kai *et al.* be withdrawn.

B. Purchio *et al.* in view of Masuda *et al.*

Claims 1-10, 14, 17-26, 33 and 35 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Purchio *et al.* (US 5,902,741) in view of Masuda." Applicants traverse this rejection.

Purchio *et al.* in combination with Masuda *et al.* fail to establish a *prima facie* case of obviousness. First, even when taken together, Purchio *et al.* and Masuda *et al.* do not teach a method requiring an engineered cartilage tissue cultured to be rapidly degraded. As set forth in Section I.A. above, contrary to the Examiner's assertion, rapid degradation of the engineered cartilage tissue of Masuda *et al.* is not an inherent feature of the tissue. Neither Purchio *et al.* nor Masuda *et al.*, alone or in combination, teach or suggest the use of engineered rapid cartilage tissue that exhibits degradation in the present screening methods. Accordingly, the references cannot render obvious the claims of the present invention. Applicants respectfully submit that because the limitation of rapid degradation in claim 1 and claim 17 is not inherent, the combination of Purchio *et al.* and Masuda *et al.* does not teach every element of the claimed invention. As claims 2-10, 14, 18-26, 33 and 35 all depend either directly or indirectly from claim 1 or claim 17, the art cited by the Examiner also fails to teach and every element of these claims. Thus, Applicants respectfully request the Examiner withdraw the rejection and allow claims 1-10, 14, 17-26, 33, and 33 to issue.

Moreover, even if "the references relied upon teach that all aspects of the claimed invention were individually known in the art it is **not** sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references." MPEP §2143.01 (emphasis added). The reasoning set forth in the Office Action is subjective reasoning that fails to show a proper motivation for one of ordinary skill in the art to combine the teachings of Purchio *et al.* with Masuda *et al.* The bare statement that "one of ordinary skill in the art would have been motivated by common culture practices in the art to culture the cartilage of Purchio *et al.* via the methods of Masuda *et al.* because it was a known method for culturing cartilage tissues," is insufficient to support the Examiner's argument.

Specifically, the meaning of "common culture practices in the art" is unclear. The skilled artisan understands that successful cell culture, especially successful mammalian cell culture, is highly dependent on a variety of factors, such as cell culture media components, temperature, pH, etc. The Examiner has failed to provide specific examples of common culture practices in the art that suggest the desirability of the combination of cited references. Thus, Applicants request that the Examiner either take official notice or provide a piece of prior art demonstrating a motivation to combine. Absent such evidentiary support to combine the references, Applicants respectfully submit that a *prima facie* case of obviousness has not been met, and the rejection must be withdrawn.

Because the prior art references neither teach or suggest every claimed element of the present invention nor do they supply motivation to combine the references, the combination of Purchio *et al.* and Masuda *et al.* cannot establish a proper *prima facie* case of obviousness. Correspondingly, Applicants respectfully request the Examiner withdraw the 35 U.S.C. § 103(a) rejection based upon the combination of Purchio *et al.* and Masuda *et al.* and allow claims 1-10, 14, 17-26, 33 and 35 to issue.

C. Saito *et al.* in view of Masuda *et al.*

Claims 1-8, 17-24, 29-30 and 35 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Saito *et al.* in view of Masuda *et al.*" Saito is cited for teaching "culturing cartilage in multi well plates in the presence of IL-1 alpha, wherein the effects of test agents are measured (p. 727)." Applicants traverse this rejection.

Yet again, a *prima facie* case of obviousness has not been satisfied as the combination of Saito *et al.* with Masuda *et al.* does not teach or suggest rapid degradation as required by independent claim 1 and independent claim 17. Nor does the Examiner provide objective evidence establishing a motivation or suggestion to combine these references or a reasonable expectation of success in light of the teachings of Saito *et al.*

As set forth above in Sections I.A. and I.B., use of an engineered tissue cultured to be rapidly degraded is not inherent to the disclosure of Masuda *et al.* Because Saito *et al.* lacks any discussion of such a tissue or procedure, Saito fails to cure this deficiency. In fact, Saito *et al.* teaches away from the present invention. By definition, the cartilage explant of Saito *et al.*

obtained directly from an animal is a non-engineered tissue. As those of ordinary skill in the art will appreciate it would be impossible to employ this "natural" tissue with the methods of Masuda *et al.* while still maintaining the essential natural character required by culture of an explant. For example, the engineered cartilage tissue used in the present invention is highly homogenous, in contrast to the "natural" cartilage tissue in cartilage explants. In some cases, such as when research centers around the response of an individual animal to a test agent, it may be more advantageous to use explant culture because intervariability between animals is irrelevant. Concerning the lack of motivation or suggestion to combine these references, Applicants request the Examiner either take official notice or provide prior art demonstrating a motivation to combine.

As the combination of Saito *et al.* with Masuda *et al.* does not satisfy the *prima facie* case requirements for demonstrating obviousness of independent claim 1 and independent claim 17, and because claims 2-8, 18-24, 29-30 and 35 all depend either directly or indirectly from claim 1 or claim 17, Applicants respectfully request that the 35 U.S.C. §103(a) rejection based on these art references be withdrawn. Applicants respectfully assert that the claims are now in condition for allowance.

D. Huch *et al.* in view of Masuda *et al.*

Claims 1-11, 17-27 and 29-30 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Huch *et al.* (1997) in view of Masuda *et al.*" This rejection must also fail for the reasons discussed above, and, in particular, those relating to the combination of Kai *et al.* and Masuda *et al.*

First, Huch *et al.* combined with Masuda *et al.* does not teach or suggest all of the claim limitations in the independent claims; namely the combination fails to teach or suggest use of an engineered cartilage matrix cultured to be rapidly degraded. In fact, the Aydelotte *et al.* reference previously submitted demonstrates that degradation of chondrocyte cells—the same type of cells used in Huch *et al.*—occurs at a much slower pace than degradation of the engineered tissue of the present invention. Aydelotte *et al.*, Articular Cartilage and Osteoarthritis 237, FIG. 2 (1992). Thus, using the cartilage of Huch *et al.* with the methods of Masuda *et al.*, in

the manner suggested by the Examiner, results in an assay with a different and slower speed of cartilage degradation that is beyond the scope of the present claims. As explained previously, rapid tissue degradation lends itself to high throughput screening, which is advantageous for many reasons.

Second, the Office Action provides no objective evidence relating to the motivation or suggestion to combine Huch *et al.* with Masuda *et al.* Again, vague assertions in the Office Action of "common culture practices" does not adequately support a suggestion or motivation to combine references. The Examiner is reminded that "the mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." MPEP §2143.01. Applicants request that the Examiner either provide official notice of the motivation to combine, provide a piece of prior art that demonstrates a motivation, or withdraw the rejection.

For the foregoing reasons, the combination of Huch *et al.* and Masuda *et al.* fails to establish a proper *prima facie* case of obviousness. Applicants respectfully request the Examiner withdraw this rejection and allow independent claim 1 and independent claim 17, as well as claims 2-11, 18-27 and 29-30 which depend therefrom, to issue.

E. Lansbury *et al.* in view of Masuda *et al.*

Finally, claims 1-8, 10, 14, 17-24, 26 and 33 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Lansbury *et al.* (WO 94/28889) in view of Masuda *et al.*" This combination also does not state a *prima facie* case of obviousness for the reasons discussed above, *e.g.*, the combination does not teach or suggest all of the elements of the claimed invention, such as the use of engineered cartilage tissue cultured to be rapidly degraded and there is no objective motivation to combine the references. Applicants request the Examiner either show motivation to combine by official notice or by providing a piece of prior art demonstrating the motivation. In light of the lack of establishment of a *prima facie* case of obviousness, Applicants respectfully request the Examiner withdraw this rejection and allow the claims to issue.

CONCLUSION

In view of the above remarks, it is respectfully submitted that this application is in condition for allowance. Early notice to that effect is earnestly solicited. The Examiner is invited to telephone the undersigned at the number listed below if the Examiner believes such would be helpful in advancing the application to issue.

Respectfully submitted,

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Inflammation Research

Glucocorticosteroids inhibit degradation in bovine cartilage explants stimulated with concomitant plasminogen and interleukin-1α

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Abstract. *Objective*: Glucocorticosteroids are beneficial in the treatment of osteoarthritis (OA) in humans, and have been shown to protect cartilage in animal models of OA. Therefore, we undertook the present study to investigate the in vitro effect of several glucocorticosteroids on cartilage degradation.
Methods: Bovine articular cartilage explants labeled with [^{35}S] Sulfate and stimulated either with IL-1α alone or with concomitant plasminogen plus IL-1α were used in this study as an in vitro model of cartilage degradation. Clobetasol propionate, fluocinolone-acetonide-21-acetate, prednisolone, triamcinolone and triamcinolone hexacetonide were the glucocorticosteroids investigated in a series of experiments, at concentrations ranging from 10 picomolar to 10 micromolar. Degradation in [^{35}S] Sulfate-labeled bovine articular cartilage explants was induced with IL-1α or with concomitant IL-1α plus human plasminogen. The effects of several glucocorticosteroids were studied, and a comparison between efficacy in explants stimulated with IL-1α alone or IL-1α plus concomitant plasminogen was made. Glucocorticosteroid efficacy was expressed as percent inhibition of degradation, and their IC_{50}s were also calculated.
Results: Glucocorticosteroids showed no protective effects on cartilage degradation in the presence of IL-1α alone. When degradation was induced by IL-1α in the presence of concomitant human plasminogen, all the glucocorticosteroids showed statistically significant inhibition ($p < 0.05$) with calculated IC_{50}s of 450–2500 picomolar.
Conclusion: The inhibition of cartilage degradation by glucocorticosteroids may be due to down-regulation of urokinase plasminogen activator (u-PA) activity. It has been shown that u-PA may be the first enzyme in the cascade of activation of pro-matrix metalloproteinases by the fibrinolytic system. Inhibition of u-PA activity may be one explanation for the efficacy of glucocorticosteroids observed in animal models of OA and with intraarticular injection in patients with OA.

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Key words: Cartilage degradation – Glucocorticosteroids – Interleukin-1 – Osteoarthritis – Plasminogen

Introduction

Intraarticular injection of glucocorticosteroids is used frequently as therapy for the treatment of osteoarthritis (OA). The residence time (or solubility) of the compounds in the articular joint space is an important factor in establishment of efficacy. Triamcinolone hexacetonide, the most insoluble glucocorticosteroid, has been shown to be the most effective. The decreased solubility may also prevent deleterious side-effects observed with high steroid concentrations [1]. The efficacy of glucocorticosteroids has clearly been demonstrated in several animal models of OA. For example, van den Berg, et al, observed steroid efficacy in young mice, despite significant inhibition of proteoglycan synthesis observed in normal mouse chondrocytes [2]. Recent studies by Pelletier et al. in a dog model of OA, show the protective effect of intraarticular injection of triamcinolone hexacetonide not only under prophylactic, but also under therapeutic conditions. The authors suggest that the effect may be mediated by direct reduction in the expression and synthesis of proteolytic enzymes, such as stromelysin (matrix metalloproteinase-3, MMP-3) and/or by inhibitory effects on cytokines/oncogenes (IL-1β, c-Fos and c-Myc) [3].

The results from in vitro studies is not consistent with the relatively well-documented in vivo activity of glucocorticosteroids. The strong direct inhibitory effect of glucocorticosteroids on cartilage degradation has never been observed in vitro. For example, Arsenis and McDonnel used IL-1-stimulated degradation in bovine and rabbit cartilage explant cultures as a model for in vivo cartilage degradation. Several antirheumatic drugs were tested in this system, including dexamethasone and prednisolone. Both compounds were essentially ineffective in inhibiting IL-1α-mediated sulfonated glycosaminoglycan (GAG) release, although some

inhibition of PGE_2 synthesis was observed [4]. IL-1α is used routinely to stimulate cartilage degradation in many in vitro models of cartilage degradation [4, 5].

We describe several in vitro studies on the effects of several popular glucocorticosteroids on recombinant human IL-1α-induced cartilage degradation in bovine articular cartilage explants under two different conditions: with and without concomitant human plasminogen, as an attempt to clarify the discrepancy between what is observed in vivo and in vitro.

Materials and methods

Recombinant human interleukin-1α was obtained from R&D Systems, Minneapolis, MN, USA. Plasminogen (from human plasma) was obtained from Athens Research and Technology, Athens, GA, USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco, Grand Island, NY, USA. [^{35}S] sulfate was obtained from Amersham, Arlington Heights, IL, USA. All glucocorticosteroids: clobetasol propionate, fluocinolone acetonide-21-acetate, prednisolone, triamcinolone and triamcinolone hexacetonide, were obtained from Sigma Chemical, St Louis, MO, USA.

Preparation of bovine articular cartilage explants

Bovine (calf) radiocarpal joints were acquired from a local abbatoir immediately after sacrifice and transported on ice. The specimens were then washed thoroughly and placed in ice containing approximately 25% Povidine (10% Povidone-iodine topical solution). The specimens were then dissected in a sterile hood using good sterile technique. Media (DMEM containing 4.5 g/L D-Glucose and L-Glutamine, without sodium pyruvate) was supplemented with HEPES buffer and sodium bicarbonate (3.57 g/L and 3.7 g/L, respectively), and the pH adjusted to 7.4. The media was further supplemented with penicillin and streptomycin (100 units/mL and 100 μg/mL, respectively) and 50 μg/mL L-ascorbic acid. The articulating cartilage surfaces were exposed, and the synovial fluid was wiped away with sterile gauze. A sterile cork-borer with a diameter of 3.5 millimeters was used to remove uniform cores of cartilage. Proper orientation was maintained by distinguishing the underlying bony layer in the cores (only the articulating surface of the cartilage cores were used in the experiments). The cores were placed in a sterile flask, washed four times with 50 mL of fresh media, and then placed in an incubator (37 °C, 5% CO_2/95% air, adequate humidity) and allowed to equilibrate for 1 h. Uniform one millimeter thick slices of the articulating surface were sliced off of each individual cartilage core using a scalpel and a specially-designed template. The cartilage disks were then labeled en mass with [^{35}S] sulfate at a concentration of 10 μCi/mL for approximately 72–96 h, with hand-stirring every few hours. After labeling, explants were equilibrated with fresh media each 48 h (minimum of two washes before use in experiments). The total time for labeling/equilibration was approximately one week from acquisition/dissection of the tissue. The slow equilibration over the course of seven days allows explants which demonstrate a lower background, thereby giving a more sensitive assay (explants used shortly after dissection label unevenly, and are in an "excited" state, giving a higher background with a diminished response to added IL-1). Explants prepared according to the method described show good viability for up to four weeks in culture. The explants used in these studies were not more than 10–15 days old (from time of acquisition).

Inhibition of IL-1-induced cartilage degradation in bovine articular cartilage in the presence of human plasminogen

For the studies described, individual explants were transferred to 96-well plates containing 250 μL of fresh media per well, with or without plasminogen and IL-1α, and with or without glucocorticosteroids. A negative control consisted of media lone, while the two positive controls were IL-1α alone, and plasminogen with IL-1α. Plasminogen by itself gave no increase in degradation over media alone (Fig. 4). All other groups in any given study contained the glucocorticosteroids along with IL-1α alone or concomitant human plasminogen plus recombinant human IL-1α. We used a sample number (n) of five, a concentration of plasminogen of 0.4 μM (physiologically relevant concentration), and an average IL-1α concentration of 0.4 ng/mL. Being that the experiments described were performed over the course of several months, two or more different batch/lot numbers of IL-1α were used. In each case, the new batch was tested (in a dose-response manner) prior to use in the experiments. The amount of IL-1α used in the various experiments ranged from 0.30 to 0.50 ng/mL, to give a similar response (approximately 20 to 35% degradation observed). The plasminogen was much more consistent from batch to batch, and there was no need to vary from a concentration of 0.4 μM in each experiment. In addition to the IL-1α and plasminogen variations, bovine cartilage specimens varied slightly from one another (due to variations in the age/size of the animals, as well as normal individual variation between specimens). A slight variation in sensitivity to added IL-1α (e.g. some specimens were more sensitive to added IL-1α) was also noted (for the same reasons stated above). In any given experiment, the cartilage from a single specimen was used (no pooling of cartilage from different specimens). It should also be mentioned that the measured degradation (or inhibition of degradation) in any given experiment is always compared to the proper control groups from that same specimen. The assay is very reproducible, and variability from specimen to specimen or different batches of IL-1α was minor.

Control and experimental explants were incubated under the conditions described for approximately 96 h (four days), prior to counting a 50 μL sample of supernatant from each well. A 50 μL sample of a papain-digest of each explant was also counted. From the counts released into the supernatant over four days, and calculation of the total counts present (determined by digest counts), the data was expressed as % GAG release over the four days. The percent GAG release value for IL-1α alone was subtracted from all other values where concomitant plasminogen and IL-1α were present, since we were only interested in plasmin/MMP-dependent degradation. The values for all groups with glucocorticosteroids present were then compared to the values for concomitant plasminogen and IL-1α without glucocorticosteroids, and percent inhibitions were calculated.

Results

IL-1α-mediated bovine articular cartilage degradation

Recombinant human IL-1α induces degradation in bovine articular cartilage explants in a dose-dependent manner (data not shown, reported previously, see reference 5). Control explants, without IL-1α, always display a basal release less than or equal to 10% of the total GAG pool labeled with [^{35}S] sulfate. Interleukin-1α at a concentration of 2.5 ng/mL initiates a two-fold increase in degradation over control, while at 10 ng/mL there is, approximately, a 35% release of [^{35}S]-labeled GAG.

IL-1α-mediated bovine articular cartilage degradation in the presence of plasminogen

Addition of human plasminogen alone to bovine cartilage explants does not induce any degradation above control (media alone) values (Fig. 4). With 1 ng/mL of IL-1α and 0.5 μM plasminogen, greater than 90% GAG release was observed (data not shown). At lower concentrations of plasminogen and IL-1α, 0.4 μM and 0.4 ng/mL, respectively, approximately 50% [^{35}S] GAG release was observed over a four-day period. We chose to use 0.4 μM plasminogen for our studies, since this is the concentration of plasminogen

found in human synovial fluid (5, and references therein). We also wanted to keep the IL-1α concentration as low as possible, and the concentrations used (0.3–0.5 ng/mL) induced submaximal stimulation and gave consistent reproducible results in the assessment of efficacy of glucocorticosteroids used in this study. The assay is very reproducible and addition of plasminogen always resulted in a three to four-fold increase in degradation over IL-1α alone. Only minor differences were observed using cartilage from different animals and different batches/lots of IL-1α.

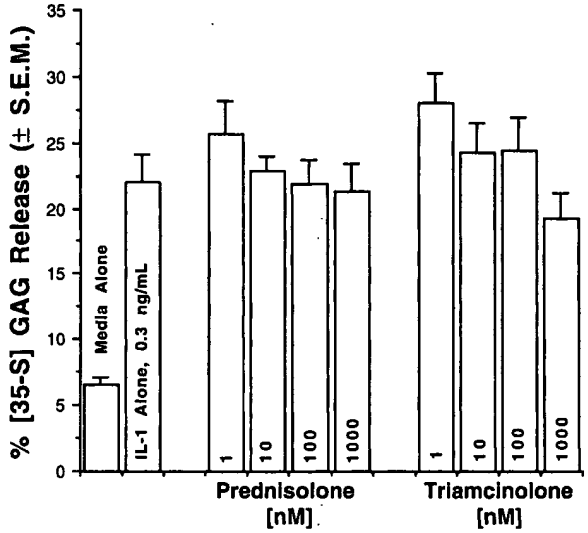

Fig. 1. Dose-responses for Prednisolone and Triamcinolone in bovine articular cartilage explants stimulated with recombinant human interleukin-1α alone (0.3 ng/mL). Doses used for each glucocorticosteroid were: 1, 10, 100 and 1000 nM, respectively. Four day assay. Error bars represent the standard error of the mean. No statistical significance was observed for any group, when compared to the control with IL-1 alone (p-values >0.05).

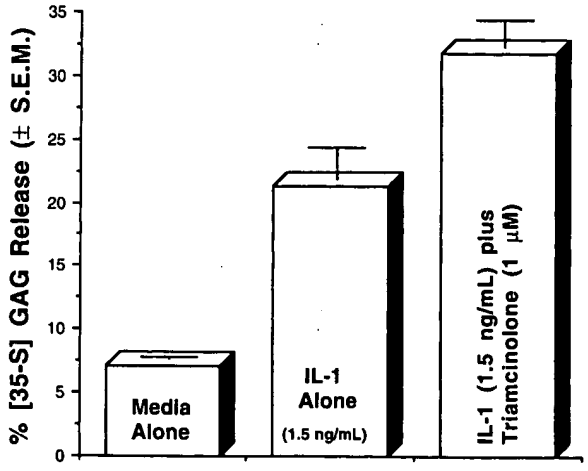

Fig. 2. Lack of inhibition of degradation by Triamcinolone (1 μM) in bovine articular cartilage explants stimulated with a high dose of recombinant human interleukin-1α alone (1.5 ng/mL). Four day assay. Error bars represent the standard error of the mean.

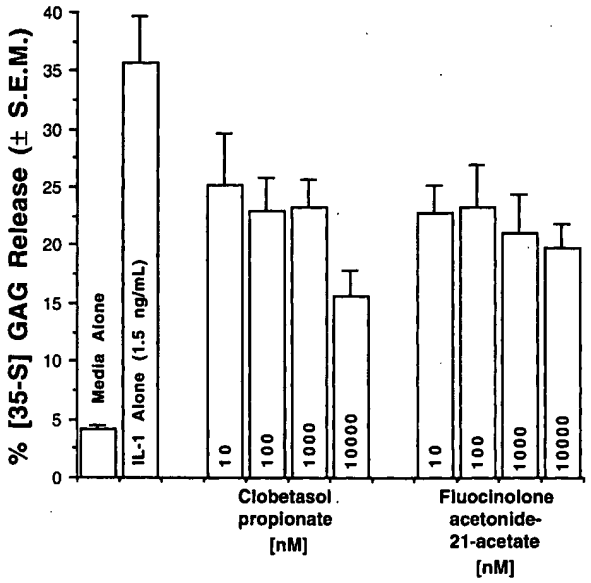

Fig. 3. Inhibition of degradation in bovine articular cartilage explants, stimulated with recombinant human interleukin-1α alone, with Clobetasol propionate and Fluocinolone acetonide-21-acetate. Doses used for both glucocorticosteroids were: 10, 100, 1000 and 10000 nM, respectively. Percentages of inhibition of degradation were as follows: 33, 40, 39, 63%; and 41, 39, 46, 50%, respectively. Four day assay. Error bars represent the standard error of the mean. All groups, except Clobetasol propionate at 10 nM, demonstrated statistical significance (p-values <0.05).

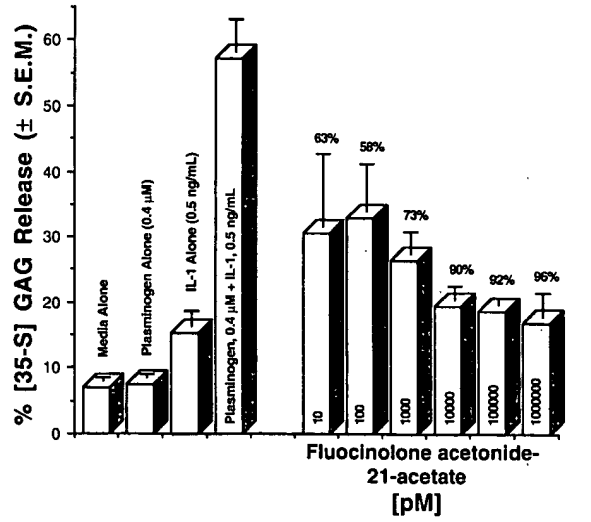

Fig. 4. Inhibition of degradation in bovine articular cartilage explants stimulated with concomitant human plasminogen (0.4 μM) and recombinant human interleukin-1α (0.5 ng/mL) by Fluocinolone acetonide-21-acetate. Percentages shown represent respective percent inhibition of degradation. Concentrations shown are in picomolar. Four day assay. Error bars represent the standard error of the mean. All concentrations from 100 pM and above demonstrated statistical significance. p-value at 10 pM was 0.0587 ("marginally significant").

Inhibition of IL-1α-induced cartilage degradation by glucocorticosteroids

An IL-1α concentration of 0.3 ng/mL stimulated 22% [^{35}S]-GAG release – this value is approximately three times above the control (media alone) release. Prednisolone and triamcinolone at a broad range of concentrations (1 nM to 1 μM) did not inhibit degradation (Fig. 1). In a separate experiment, where a higher concentration of IL-1α was used (1.5 ng/mL), triamcinolone at a concentration of 1 μM resulted in stimulation of [^{35}S]-GAG release in our model (Fig. 2). Under similar conditions, a concentration of 1.5 ng/mL of IL-1α induces about 35% release of [^{35}S]-GAG (Fig. 3). Both clobetasol propionate and fluocinolone acetonide-21-acetate demonstrated statistically significant inhibition with a broad range of concentrations (10 nM to 10000 nM), however, a dose-response relationship is not observed. The efficacy observed with clobetasol propionate at 10 μM (63% inhibition) could be due to toxic effects of the very reactive chloromethylketone moiety.

Inhibition of IL-1α-induced cartilage degradation by glucocorticosteroids in the presence of concomitant plasminogen

In the presence of human plasminogen at a concentration relevant in vivo, 0.4 μM [5], 0.5 ng/mL of IL-1α induces almost 60% [^{35}S]-GAG release. Under these conditions, fluocinolone acetonide-21-acetate is a very effective inhibitor of cartilage degradation, and statistically significant inhibition is observed at 100 pM (Fig. 4). Inhibition for all the glucocorticosteroids investigated is dose-dependent in our concomitant plasminogen/IL-1α model, and the demonstrated IC_{50}'s are below 1 nM – with the single exception of triamcinolone hexacetonide, for which the IC_{50} was 2.5 nM (Table 1). Examples of glucocorticosteroid dose-response curves are shown in Fig. 5.

Table 1. Inhibition of [^{35}S]-GAG release by glucocorticosteroids, in bovine articular cartilage explants stimulated with concomitant human plasminogen (0.4 μM) and recombinant human IL-1α (average of 0.5 ng/mL).

Glucocorticosteroid	IC_{50}* [pM]
Clobetasol propionate	450
Fluocinolone acetonide-21-acetate	600
Prednisolone	600
Triamcinolone	300
Triamcinolone hexacetonide	2500

* Concentration at which fifty percent inhibition is observed (picomolar).

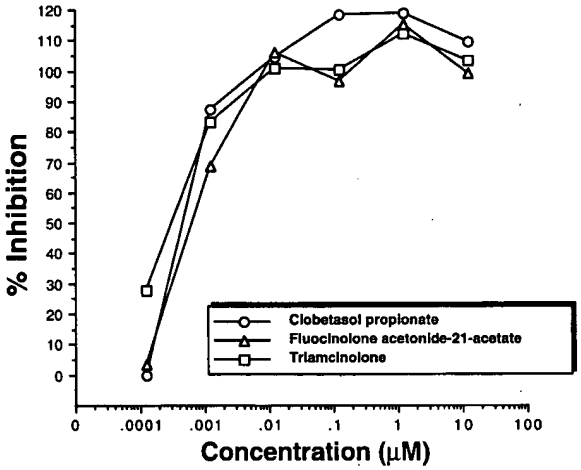

Fig. 5. Dose-response inhibition curves for three glucocorticosteroids: Clobetasol propionate, Fluocinolone acetonide-21-acetate and Triamcinolone, with concomitant human plasminogen (0.4 μM) and human interleukin-1α (0.5 ng/mL). Four day assay.

Discussion

Glucocorticosteroids are strong antiinflammatory and immunosuppressive agents. The mechanism of action is still poorly understood, but it is generally accepted that they form complexes with some members of the steroid hormone-receptor superfamily. Such complexes are responsible for "silencing" of early-response genes important in the inflammatory process [6]. Recently, Auphan, et al, reported that glucocorticosteroids are potent inhibitors of nuclear factor kappa B (NF-κB) activation, both in cultured cells and mice [7]. NF-κB plays a central role in the induction of a large number of immunoregulatory genes, including those encoding: IL-1, IL-2, IL-3, IL-6, IL-8, TNF-α, interferon γ, granulocyte-macrophage colony-stimulating factor (GMCSF), class-I and class-II major histocompatibility complex, the κ light chain, endothelial leukocyte adhesion molecule-I and intercellular adhesion molecule-I. Several of these genes are also regulated by AP-1, which synergizes with NF-κB (7, and references therein). Consistent with these properties, intra-articular injection of glucocorticosteroids as therapeutics for osteoarthritis, provides beneficial effects in humans [1], and effective protection of cartilage has been demonstrated in several animal models of cartilage pathology [2, 3]. Many of the earlier studies used very high doses of oral steroids or repeated local injection, and it is now known that these practices may actually worsen OA cartilage lesions [8].

In contrast to the good efficacy of glucocorticosteroids observed in vivo, the direct inhibition of GAG degradation in vitro has never been demonstrated. Indirect evidence of protection, such as evidence for the inhibition of synthesis of proteolytic enzymes [3, 9], or stimulation of synthesis of tissue inhibitor of metalloproteinase (TIMP) have been demonstrated [9]. However, when proteoglycan release was monitored, either no effect [4] or very weak inhibition was observed (see Results section).

We report here a very potent activity for glucocorticosteroids. Cartilage protection could only be demonstrated in vitro when a physiologically relevant concentration of human plasminogen (same concentration as found in human synovial fluid) was added to the assay. Recently, we reported that in the presence of plasminogen, both bovine and human articular cartilage become very sensitive to the effects of added IL-1 [5]. Concentrations of IL-1α of less than 1 ng/mL

were observed to induce significant degradation. With human cartilage explants, very little degradation is observed with IL-1 when plasminogen is not present [5].

Stimulated chondrocytes are known to both produce and activate urokinase plasminogen activator (u-PA). u-PA can activate plasminogen to plasmin, which is a well-known pro-MMP activator. Prior studies with human articular cartilage explants suggest that this pathway could be the dominant pathway of degradation in human cartilage (5, and references therein). Immunohistochemical studies show the presence of both u-PA and plasmin in human OA cartilage. Moreover, a statistically significant correlation was found between plasmin activity and the free collagenolytic form in human OA articular cartilage specimens [10].

u-PA activity represents a crucial first step in the MMP-activation cascade. Plasminogen activator inhibitor-1 (PAI-1) is a very effective inhibitor of human cartilage degradation in vitro, with an IC_{50} below 100 nM [5]. Some u-PA and plasmin inhibitors have demonstrated good efficacy in vivo. Transexamic acid [11] and recombinant human protease nexin-1 [12] are active inhibitors of cartilage degradation in animal models, and urinary trypsin inhibitor was reported to be efficacious in human patients with OA [13].

Glucocorticosteroids have been reported to reduce plasminogen activator activity in a variety of cell-types and tissues [14]. In human chondrocytes, dexamethasone suppressed IL-1 and TNF-α-mediated induction of u-PA activity [15]. Thus, it seems that the potent effect of glucocorticosteroids on cartilage protection in vitro during concomitant plasminogen/IL-1 stimulation could be due to suppression of u-PA activity. The in vivo efficacy reported for u-PA/plasmin inhibitors [11–13] demonstrates the importance of the fibrinolytic system (u-PA/Plasminogen/Plasmin) as an activator of up-regulated pro-MMPs [16].

We should not underestimate the antiinflammatory and immunosuppressive properties of glucocorticosteroids, especially regarding suppression of synthesis of IL-1 and TNF-α (these cytokines also induce more u-PA activity), or TIMP-3 mRNA expression stimulated by dexamethasone [17]. However, in vivo, the system does possess a limited number of "tools" (proteolytic enzymes) to degrade extracellular matrix. The data presented here provides additional lines of evidence that the fibrinolytic system could be responsible for the activation of MMPs observed in cartilage degradation under pathological conditions, like OA. The glucocorticosteroids may inhibit this cascade in vivo, simply by diminishing the plasminogen activator supply. Our data suggests that therapeutically effective concentrations should be in the very low nanomolar range. The intraarticular injection of glucocorticosteroids, especially those with very low solubility, or those that are released slowly over time, should minimize the systemic side-effects observed with these compounds. Clearly, glucocorticosteroids as therapeutic agents for OA deserve more attention from clinicians.

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